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Haemocin, the Bacteriocin Produced by *Haemophilus influenzae*: Species Distribution and Role in Colonization

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Four hundred thirty-eight strains of *Haemophilus influenzae* were examined for production of and sensitivity to haemocin, a bacteriocin produced by some members of this species. Whereas 199 of 212 (94%) type b isolates produced haemocin, 131 of 134 (98%) nontypeable and 91 of 92 (99%) encapsulated non-type b isolates were sensitive to haemocin. Among strains previously genetically characterized by multilocus enzyme electrophoresis, haemocin production was detected in type b isolates belonging to 25 of 29 (86%) clonally distinct electrophoretic types. None of 60 clonally distinct nontypeable strains produced this substance, and all were sensitive to it in vitro. The genes encoding haemocin production were transformed independently of the genes necessary for capsule expression from a prototypic type b strain to a nontypeable strain. After intranasal inoculation of infant rats with an equal mixture of a non-haemocin-producing strain and its haemocin-producing transformant, organisms capable of haemocin production predominated in both nasopharyngeal and blood cultures. These data demonstrate that haemocin production is strongly associated with type b encapsulated members of this species and suggest a mechanism by which haemocin might play a role in host nasopharyngeal colonization by this pathogen.

Haemophilus influenzae is a human-specific pathogen (33). Nonencapsulated (serologically nontypeable) and encapsulated non-type b strains are relatively avirulent. However, strains with the type b capsule cause a variety of invasive human infections including meningitis, pneumonia, septic arthritis, cellulitis, and epiglottitis (4). Whereas the type b polysaccharide, polyribosyl ribitol phosphate, has been extensively investigated and shown to be an important virulence factor, other features of *H. influenzae* type b such as pili (1), lipooligosaccharides (12), immunoglobulin A proteases (11), and certain outer membrane proteins (OMPs) (32) may also contribute to the virulence of this bacterium.

In 1975, Venezia and Robertson described a bactericidal factor, haemocin, which was produced by type b encapsulated *H. influenzae* and was active against other *Haemophilus* species (35). Preliminary characterization of this substance suggested that it was an extracellular protein similar in size and mechanism of action to other bacteriocins.

The purpose of our study was to define more precisely the distribution of this phenotype within the *H. influenzae* species and to investigate the potential role of haemocin in the pathogenesis of infection due to *H. influenzae* type b.

MATERIALS AND METHODS

Bacteria. Serotype b, haemocin-producing *H. influenzae* E1a and serologically nontypeable, haemocin-sensitive strain Rd have been described previously (3, 29). Strain R55 (KW-20), a serologically nontypeable derivative of a type d parent, and its serotype b transformant, R56 (KW-20b), have also been described (17). Additional *H. influenzae* isolates were kindly contributed by J. M. Musser, University of Rochester, Rochester, N.Y.; J. R. Gilsdorf, University of Michigan, Ann Arbor; and S. J. Barenkamp, Washington University, St. Louis, Mo., or were obtained from the clinical microbiology laboratory of St. Christopher's Hospital for Children, Philadelphia, Pa. Isolates were stored in sterile skim milk at −70°C as described before (24).

Haemocin assay. Production of haemocin by *H. influenzae* was detected by using an in vitro bioassay. Approximately 10^5 CFU of strain Rd were swabbed onto brain heart infusion agar supplemented with 10 μg of hemin and 10 μg of β-NAD per ml (sBHI agar). This lawn was overstreaked with the *H. influenzae* test strain, and after overnight incubation at 37°C, a clear zone surrounding the test strain was indicative of haemocin production. Conversely, sensitivity to haemocin was detected by inhibition of the test strain by *H. influenzae* E1a.

Haemocin preparation. Crude haemocin was prepared as described previously (34). In brief, proteins were precipitated with 55% ammonium sulfate from the cell-free sBHI broth culture supernatant obtained after overnight growth of E1a. The precipitate was suspended in 50 mM Tris hydrochloride (Tris buffer; pH 7.4) to 4% of the original volume and dialyzed exhaustively against the same buffer. The total protein content of this crude preparation was determined by the method of Peterson (20), and proteins were analyzed by electrophoresis in a sodium dodecyl sulfate (SDS)-polyacrylamide (15%) gel stained with Coomassie brilliant blue (28). "Mock" haemocin was prepared in the same manner from broth culture supernatant obtained from overnight growth of strain Rd.

Haemocin bactericidal activity. *H. influenzae* R55 was grown to mid-log phase in sBHI broth, washed once with phosphate-buffered saline (PBS; pH 7.2), and suspended to a concentration of 10^7 CFU/ml in sBHI broth. Equal amounts of this suspension and crude haemocin were mixed and allowed to incubate at room temperature. Control samples of R55 suspended in sBHI broth were mixed with equal volumes of either Tris buffer or mock haemocin and also incubated at room temperature. Colony counts were performed on timed aliquots of these mixtures.

DNA preparation. Chromosomal DNA was purified from organisms grown to stationary phase by chloroform-phenol

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TABLE 1. Distribution of haemocin production and sensitivity among 438 *H. influenzae* isolates

Isolates	No. of haemocin producers (%)	No. of haemocin nonproducers (%)[a]	
		Hmc-S	Hmc-R
Type b	199 (93.8)[b]	12 (5.7)	1 (0.5)
Nontypeable	2 (1.5)[c]	131 (97.8)	1 (0.7)
Types a, c–f	0	91 (98.9)	1 (1.1)

[a] Hmc-S, haemocin sensitive; Hmc-R, haemocin resistant.
[b] $P < 10^{-8}$ (Fisher's exact test); haemocin production versus nonproduction of type b versus non-type b.
[c] Two nontypeable strains probably derived from type b strains as described in the text.

extraction of whole-cell lysates followed by ethanol precipitation, as described previously (27).

Transformation. Bacteria were grown to early log phase in sBHI broth and made competent in M-IV media as described by Herriot et al. (9). Competent cells (approximately 10^9 CFU) were incubated in the presence of 1 μg of purified chromosomal DNA for 30 min at 37°C. Transformants were grown in sBHI broth for 2 h before selection by exposure to crude haemocin. Bacteria surviving the selective step were diluted, subcultured, and screened for haemocin production and sensitivity. Certain transformants were also screened for type b capsule production by halo formation on sBHI agar supplemented with burro anti-polyribosyl ribitol phosphate antisera as described before (14).

OMP and lipooligosaccharide analysis. OMPs were prepared from sonicated mid-log-phase organisms by extraction with Triton X, resolved by electrophoresis in an SDS-polyacrylamide (15%) gel, and stained with Coomassie brilliant blue as described previously (28). Lipooligosaccharide analysis was performed by gradient SDS-polyacrylamide gel electrophoresis as described by Kimura and Hansen (12).

Infant rat studies. Newborn pups, delivered of timed-pregnant Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind.), were pooled, randomly reassigned to the mothers, and maintained in cages fitted with spun-polyester filter hoods in a biocontainment facility to prevent cross-contamination. At 5 days of age, rats were intranasally inoculated with bacteria which had been grown to late log phase, washed once, and suspended to the desired concentration in PBS supplemented with 0.1% gelatin. At 48 h after inoculation, nasopharyngeal cultures were obtained by instilling 20 μl of sterile PBS into one naris and culturing 10 μl of the fluid obtained from the contralateral naris onto sBHI agar containing vancomycin, bacitracin, and clindamycin (7). After overnight incubation, at least 20 colonies from each culture were tested for haemocin production. A 10-μl portion of blood was obtained via tail vein or cardiac puncture at 5, 8, 11, 14, 17, and 21 days after intranasal inoculation and cultured on sBHI agar. Resultant colonies were also analyzed for haemocin production as described above.

RESULTS

Haemocin production by *H. influenzae*. A total of 438 *H. influenzae* isolates were blindly screened for haemocin production and sensitivity (Table 1). Haemocin production was detected in 168 (93%) of 180 type b isolates which had been collected from 13 different countries on five continents and previously genetically characterized by multilocus enzyme electrophoresis (19). A subset of these type b isolates had been analyzed previously for their genetic distribution within the *Haemophilus* species (18); strains belonging to 25 (86%) of 29 unique electrophoretic types (ETs) produced haemocin, whereas those from the other 4 ETs did not. Only strains representing genetically highly divergent type b ETs failed to produce haemocin. Some 93% of these 180 type b strains had been isolated from blood or cerebrospinal fluid culture.

To assess the possibility that haemocin production is related to the site from which the organism is recovered, 29 additional type b isolates cultured from mucosal sites from children with ($n = 14$) and without ($n = 15$) invasive disease were investigated. Of these strains, 28 (96%) produced haemocin.

Among the 134 serologically nontypeable *H. influenzae*, 60 had been characterized previously by multilocus enzyme electrophoresis and found to represent 60 unique ETs (18). None of these 60 strains produced haemocin, and all were sensitive to it in vitro. An additional 45 nontypeable isolates obtained from cultures of mucosal sites or sputum as well as 26 isolates cultured from blood or cerebrospinal fluid also failed to produce haemocin, and all except one were sensitive to it.

A total of 92 encapsulated non-type b isolates (26 type a, 9 type c, 23 type d, 26 type e, and 8 type f) were also examined. None of these produced haemocin, and only one was resistant to haemocin in vitro.

Finally, three pairs of isolates reported previously by Hoiseth and Gilsdorf (10) were investigated. These isolates were obtained from three patients who had systemic disease due to a type b strain and who also had a nontypeable strain isolated from the nasopharynx during the course of their antibiotic therapy. To confirm the absence of type b capsule, the nasopharyngeal isolate in each case was serotyped by using commercial type b antisera and examined by latex agglutination assay for the presence of type b capsular polysaccharide. These investigators also analyzed all six isolates by OMP profiles and by Southern hybridization with a DNA probe containing genes required for type b capsule expression (Cap b probe). We blindly screened these isolates for haemocin production and sensitivity. All three type b isolates and two of the nontypeable isolates produced haemocin. These two nontypeable isolates were found by Hoiseth and Gilsdorf to have OMP profiles identical to those of their corresponding type b isolates and to hybridize with the Cap b probe, suggesting that they were derived from the type b encapsulated strain. The remaining nontypeable isolate demonstrated an OMP profile different from its corresponding type b isolate and did not hybridize to the Cap b probe; this isolate did not produce haemocin in vitro.

Haemocin bactericidal activity. The crude haemocin preparation had a total protein content of approximately 2 μg/μl and resolved as a diffuse, intensely staining band near the dye front of an SDS-polyacrylamide (15%) gel. The survival of R55 in the presence of this preparation was examined and compared with R55 incubated with either Tris buffer or the mock haemocin preparation. When 10^7 CFU/ml was mixed with an equal volume of crude haemocin, rapid killing of R55 occurred, with a 3-log reduction in viable bacteria within 90 min (Fig. 1). In contrast, no killing was detected in the control samples incubated with Tris buffer or the mock haemocin. Further, no killing of strain E1a by the crude haemocin preparation was detected (data not shown).

Transformation. The killing capacity of haemocin was utilized for selecting haemocin-resistant transformants. Strains R55 and R56 were transformed with chromosomal DNA purified from strain E1a. After transformation, bacte-

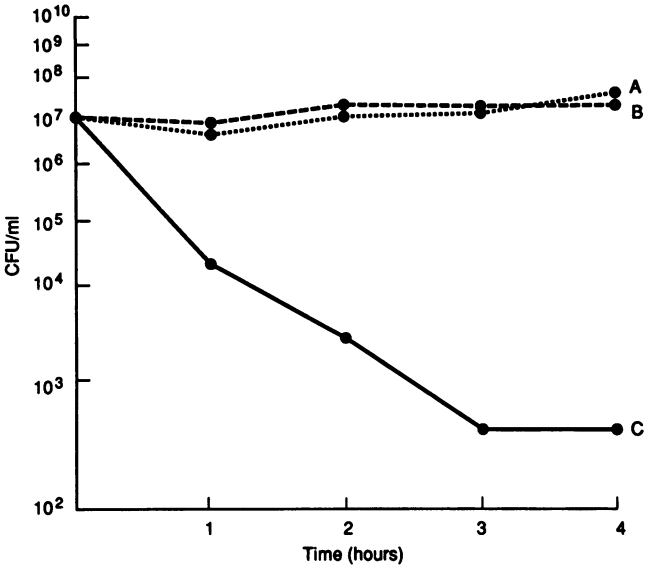

FIG. 1. Survival of *H. influenzae* R55 after exposure to Tris buffer (A), mock haemocin (B), or crude haemocin (C) in vitro.

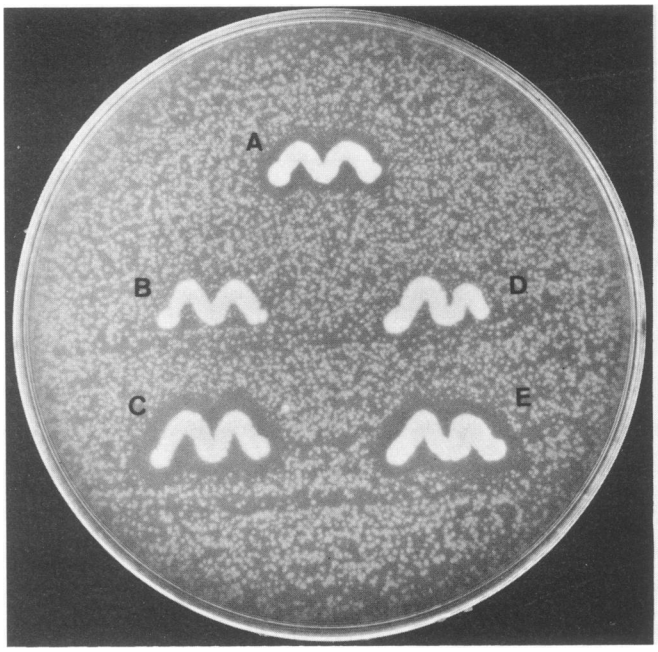

FIG. 2. In vitro bioassay for haemocin production. A lawn of haemocin-sensitive strain Rd was overstreaked with strains E1a (A); R55 (B); the haemocin-producing transformant of R55 (C); R56 (D); and the haemocin-producing transformant of R56 (E).

ria were incubated with crude haemocin for 90 min. Among the surviving bacteria, haemocin-producing transformants as well as mutants resistant to haemocin, but not producers of it, were found in a ratio of approximately 1:5. Haemocin-resistant nonproducers were also detected after exposure of strains R55 and R56 to crude haemocin in the absence of E1a DNA. Haemocin activity was fully expressed in the haemocin-producing transformants as determined by a comparison of the zones of inhibition produced by these transformants and *H. influenzae* type b E1a (Fig. 2). None of 20 haemocin-producing transformants of R55 produced type b capsular polysaccharide as determined by the absence of halo formation on sBHI agar containing anti-polyribosyl ribitol phosphate antisera. One type b encapsulated, haemocin-producing transformant of strain R56 (R56P) was chosen for study in the infant rat model as described below.

OMP and lipooligosaccharide analyses. Strains R55 and R56 and their respective haemocin-producing transformants and haemocin-resistant, nonproducing mutants did not differ in their OMP or lipooligosaccharide profiles (Fig. 3).

Infant rat studies. Atraumatic intranasal inoculation of 10^6 CFU of either strain R56 or its haemocin-producing transformant, R56P, produced bacteremia in infant rats at relatively low rates; 5 of 19 (26%) and 8 of 23 (35%) rats became bacteremic, respectively. The difference between these rates is not significant ($P > 0.05$). All colonies examined from rats inoculated with R56 did not produce haemocin when tested in vitro, whereas those from rats receiving R56P produced haemocin without exception.

In another series of experiments, equal quantities of strains R56 and R56P were mixed in PBS supplemented with 0.1% gelatin immediately before intranasal inoculation of 10^6 CFU into infant rats. Control samples of this mixture were allowed to incubate at room temperature for 30 min and cultured onto sBHI agar; after overnight incubation, they yielded approximately equal numbers of haemocin-producing and haemocin-nonproducing colonies. At 48 h after inoculation, *H. influenzae* was cultured from the nasopharyngeal washings of 17 of 22 rats; all of these were pure cultures of haemocin-producing organisms. None of these rats developed bacteremia.

When 10^7 to 10^8 CFU of an equal mixture of haemocin-producing and -nonproducing organisms were inoculated intranasally, 10 of 22 (45%) rats developed bacteremia during the following 3 weeks. The peak incidence of bacteremia was on day 17 after inoculation, at which time 9 of 10 rats yielded pure cultures; 7 of these 9 were pure cultures of the haemocin-producing strain, whereas the remaining 2 rats yielded only haemocin-nonproducing colonies. Overall, haemocin-producing organisms predominated among bacteria recovered from the bacteremic rats on each of the days examined; the mean daily proportion of haemocin-producing colonies obtained from all bacteremic rats ranged from 76 to 82% during the 3 weeks after inoculation.

Nasal washes yielded pure cultures (or a 20-fold excess of one or the other variant) from 7 of 21 rats colonized 48 h after inoculation of 10^7 to 10^8 CFU of the R56-R56P mixture (Fig. 4). In a control experiment, only 1 of 9 rats receiving an intranasal inoculation of an equal mixture of strain R56 and a nalidixic acid-resistant mutant of R56 yielded a pure culture after 48 h. The mean proportion of haemocin-producing colonies obtained at 48 h from rats inoculated with the R56-R56P mixture was 86% (95% confidence interval, 81 to 92%). In contrast, the mean proportion of nalidixic acid-sensitive colonies recovered from rats receiving the mixture of R56 and its nalidixic acid-resistant mutant was 59% (95% confidence interval, 43 to 76%). The difference between these proportions is significant ($P < 0.001$, Student's t test).

DISCUSSION

Bacteriocins are proteins that are elaborated by a variety of bacterial species and are active against certain strains of the homologous or related species (8, 13, 31). These substances have been found to vary considerably in structure, host range, mechanism of action, and genetic basis. Al-

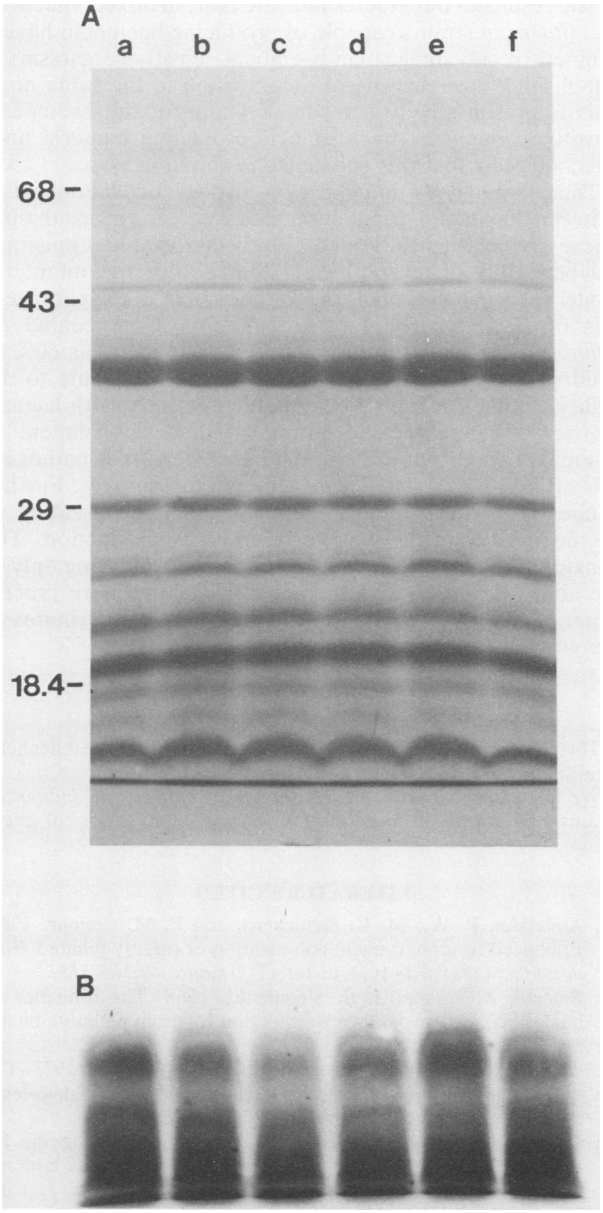

FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of OMP (A) and lipooligosaccharides (B) from *H. influenzae* R55 (lane a), R56 (lane d), their respective haemocin-producing transformants (lanes b and e), and haemocin-resistant mutants (lanes c and f).

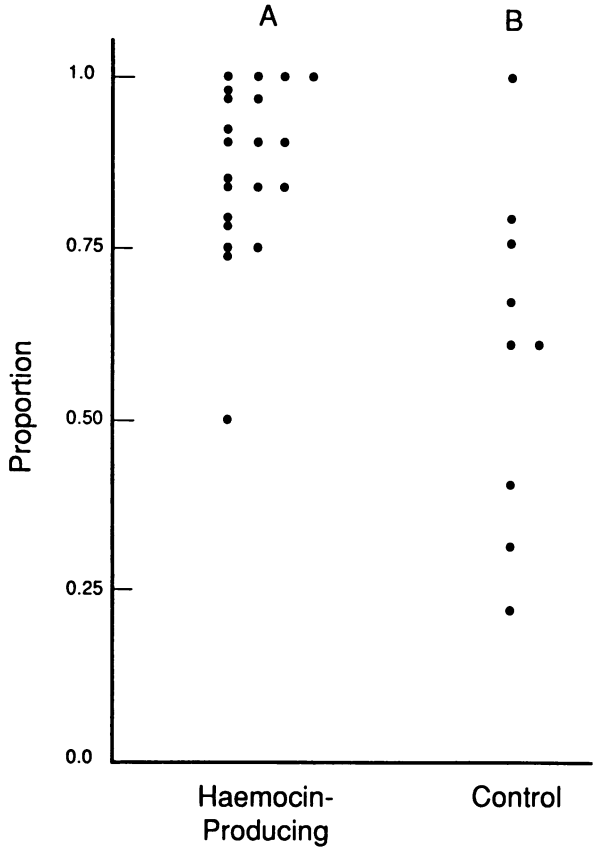

FIG. 4. Proportion of haemocin-producing organisms recovered from nasal culture 48 h after inoculation of an equal mixture of haemocin-producing and -nonproducing *H. influenzae* (A). Control indicates proportion of nalidixic acid-sensitive organisms recovered from nasal culture 48 h after inoculation of an equal mixture of nalidixic acid-sensitive and -resistant *H. influenzae* (B).

though they have been identified in numerous genera, bacteriocins have not been found in certain human-specific pathogens such as *Neisseria*. Several investigations have suggested that bacteriocins may be factors involved in the pathogenesis of certain infectious diseases (2, 22, 26). Other studies have demonstrated that some bacteriocins may be cytotoxic to eucaryotic cells (5, 6, 23). However, the exact role of these substances in the pathogenesis of infectious disease or in microbial ecology remains unclear (25).

Haemocin, the term applied to the bacteriocin produced by *H. influenzae*, has not been extensively studied. Venezia and Robertson demonstrated that haemocin was produced by all 28 type b *H. influenzae* they examined (35). Also, none of 32 nonencapsulated or 8 encapsulated non-type b isolates produced the substance, and all were susceptible to it in vitro. Varying proportions of other *Haemophilus* species as well as various other gram-negative bacteria also demonstrated sensitivity to haemocin. Stuy found haemocin activity among 53 of 56 type b isolates (30). These observations, like those regarding the frequency with which other phenotypes, e.g., pili (1) and immunoglobulin A protease (11), are expressed among *Haemophilus* strains, are limited by their reliance on an analysis of strains of unknown genetic relatedness. To assess the distribution of the haemocin phenotype among *H. influenzae* more accurately, we examined previously characterized strains known to represent the breadth of genetic diversity within this species (18). Haemocin production was detected among 86% of the type b strains found to be clonally distinct by ET. The only type b strains which did not produce haemocin were those found among genetically highly divergent ETs (ETs 48, 87, 88, and 89 in reference 17). Interestingly, however, certain other closely related type b strains (representing ETs 46, 47, and 90) did produce haemocin. Haemocin production was not found among 60 clonally distinct nontypeable *H. influenzae* strains; all of these were sensitive to haemocin in vitro. The high degree of evolutionary conservation of haemocin production among the serotype b *Haemophilus* strains suggests that this factor plays a role in the biology of this human-specific pathogen.

Haemocin production and sensitivity were not related to the site from which clinical isolates of *H. influenzae* were

obtained. Type b isolates cultured from both invasive (blood and cerebrospinal fluid) and mucosal sites produced haemocin. In contrast, nontypeable isolates obtained from mucosal as well as invasive sites were sensitive to haemocin. Further, these phenotypes remained stable after multiple passages in vitro (data not shown). Thus, it is unlikely that haemocin production is merely a function of site-dependent phase variation among *Haemophilus* strains.

Only two nontypeable isolates were found to produce haemocin. Based on OMP profile and Southern hybridization, both isolates were most likely derived from type b parents. Venezia and Robertson similarly reported that nonencapsulated mutants of five type b strains (Rb strains) produced haemocin (35). Our finding that the genes encoding haemocin production transformed independently of the genes specifying type b capsule production confirms similar observations by Venezia et al. (34). These data suggest that, although haemocin production is closely correlated with type b encapsulation, the genes encoding these two phenotypes are not closely linked.

In light of the clear association of haemocin production with type b encapsulated strains of *H. influenzae*, we undertook preliminary studies using the infant rat model to explore the potential role of haemocin in the pathogenesis of infection by this bacterium. Although the rate with which haemocin-producing organisms produced bacteremia in infant rats was slightly greater than that produced by the nonproducing parent strain, this difference was not statistically significant. However, both rates were less than those expected with wild-type b organisms (7, 17) and comparable to the rate observed by Moxon and Vaughn when they used the same type b transformant in the infant rat model (17). Studies that use isogenic wild-type b strains differing only in their ability to produce haemocin may demonstrate differences in invasive capacity not apparent with the type b transformants used in the present study.

When 10^6 CFU of an equal mixture of the haemocin-producing transformant and its nonproducing parent were inoculated into infant rats, only the haemocin-producing transformant was obtained from subsequent nasal wash culture. When the mixture inoculum was increased to 10^7 to 10^8 CFU, the proportion of rats whose nasopharyngeal secretions had pure cultures (or a 20-fold excess of one or the other phenotype) decreased, although 33% of rats still had pure cultures of the haemocin-producing transformant. Further, the majority (86%) of organisms recovered from all colonized rats 48 h after inoculation were haemocin producing. In contrast, the predominant phenotype cultured from a control group receiving a mixture of strains expected to be equally virulent, i.e., parent strain R56 and its nalidixic acid-resistant mutant, was represented in only 59% of the organisms recovered at 48 h ($P < 0.001$, Student's t test).

These findings are in striking contrast to previously reported studies regarding the dynamics of nasopharyngeal colonization by mixed populations of *H. influenzae* type b in the infant rat model. Moxon and Murphy reported that all nasopharyngeal cultures produced mixed populations when obtained 24 h after intranasal administration of inocula of $>10^5$ bacteria composed of an equal mixture of streptomycin-sensitive and streptomycin-resistant variants of *H. influenzae* type b strain Eagan (15). Rubin and Moxon reported similar findings with very low inocula of mixed populations of *H. influenzae* type b; as the inoculum of 50% mixtures of two antibiotic resistance phenotypes was increased from 10^2 to 1.2×10^3 CFU, the proportion of pure nasopharyngeal cultures obtained at 48 to 72 h decreased from 85 to 40% (21).

The results of our studies indicate that, in mixed infection, *H. influenzae* strains capable of producing haemocin have a competitive advantage over haemocin-sensitive organisms in establishing nasopharyngeal colonization in the same host. This is presumptively a result of killing of the haemocin-sensitive organisms by haemocin-producing bacteria and, thus, suggests that this substance is active in vivo.

Thus, among *H. influenzae*, a strong correlation exists between haemocin production and type b encapsulation. These phenotypes, although closely associated, transform independently. The results of studies with the infant rat model suggest a potential role for haemocin in the pathogenesis of *H. influenzae* type b infection. Nontypeable *H. influenzae* colonize the nasopharynx in as many as 60% of children (4). Haemocin production might contribute to the ability of type b strains to compete effectively with haemocin-sensitive nontypeable strains in the establishment of nasopharyngeal colonization, the initial step in the pathogenesis of invasive *H. influenzae* type b infection (16). Further studies will focus on the biochemical characterization of haemocin and on the genetic basis of its production. The construction of isogenic wild-type b strains differing only in the ability to produce this protein will allow more precise experiments to determine whether haemocin contributes to the virulence of this important human pathogen.

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